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The EvA study: aims and strategy

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Abstract

The EvA study is an EU funded project (# 200506) under Frame Work Program 7 (FP7), which aims at defining new markers for COPD and its subtypes. The acronym is derived from **e**mphysema **v**ersus **a**irway disease, indicating that the project targets these two main phenotypes of the disease. The EvA study is based on the concept that emphysema and airway disease are governed by different pathophysiological processes and these are driven by different genes and a differential gene expression in the lung. To define these genes patients and non-COPD controls are recruited for clinical examination, lung function analysis and computed tomography of the lung. CT scans are used to define the phenotypes based on lung density and airway wall thickness. This is followed by bronchoscopy in order to obtain samples from the airways and the alveoli. These tissue samples along with blood samples are then subjected to genome-wide expression and association analysis and markers linked to the phenotypes are identified. The population of the EvA study is different from other COPD study populations, since patients with current oral glucocorticoid, antibiotics, exacerbations or current smoking are excluded, such that the signals detected in the molecular analysis are due to the distinct inflammatory process of emphysema and airway disease in COPD.

Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by an airflow limitation that is not fully reversible [1]. COPD is an inflammatory disease, which shows gradual progression over time and periods of acute deteriorations (exacerbations) will lead to further decline in lung function [2]. Inflammatory changes occur in the proximal and distal airways and in the lung parenchyma and here increased numbers of leukocytes like neutrophils, macrophages and CD8 lymphocytes can be found [3]. These leukocytes and the mediators they release (e.g. cytokines and proteases) lead to tissue remodelling and destruction of airways and parenchyma [4].

The disease is caused by smoke inhalation either from cigarettes [5, 6] or from exposure to other noxious particles such as those from open fires used for cooking or heating in poorly ventilated homes [7, 8]. In addition to these environmental triggers, the development of the disease is controlled by genes as suggested by the finding that only about 20% of smokers succumb to COPD [9] and by the observation that the disease runs in families [10, 11]

In the quest to define the genes involved, large numbers of patients have been studied for association of COPD with genetic markers as defined by SNP genotyping using blood leukocyte DNA. These studies have revealed association with several candidate loci like chromosome 4q31 locus (HHIP), chromosome 4q22 locus (FAM13A) and chromosome 15q25 locus (CHRNA3/CHRNA5/IREB2) as summarized in Silverman et al [12] but it remains unclear whether and how the respective genes encoded in these loci are involved in the pathophysiology of the disease.

One of the difficulties in identifying relevant markers associated with COPD is that COPD is not one entity but it is a heterogeneous disease with different phenotypes. While fixed airway obstruction as assessed by postbronchodilator FEV1 is the hallmark of COPD this airway obstruction can arise by different mechanisms. One is due to inflammatory destruction of the alveoli and of elastic fibres leading to emphysema. Here the airways collapse because they are not properly embedded in and are not kept suspended by the surrounding lung tissue. Another mechanism is more direct and is due to narrowing of airway lumen because of inflammatory thickening of the airway walls, a process termed bronchitis or airway disease.

The EvA project is based on the concept that many patients with COPD show both emphysema and airway disease but others present with a predominance of either emphysema (E) versus airway disease (A). These phenotypes may have different clinical courses, they may respond differently to therapy and importantly they may be controlled by different genes. Therefore it is important to define emphysema and airway disease by robust technology.

Computed tomography (CT) of the lung has emerged as one such technology. The use of lung CT for assessment of emphysema was pioneered in studies by Hayhurst et al [13]. Here the degree of emphysema is reflected in the reduction of lung density and digital image analysis of whole lung scans is now available to express this in Hounsfield units. On the other hand, the degree of airway disease is reflected in a thickening of the airway wall and this can be determined as percentage of the airway wall area relative to the entire airway area from appropriate CT scans. It is the obstruction of the small airways (< 2 mm internal diameter) that is relevant to the increased airway resistance in COPD [14,15] but assessment of the dimensions for these small airways is beyond resolution of CT.

However, Nakano et al demonstrated that the dimension of proximal airways as assessed by CT correlates with the dimensions of small airways as assessed in histology [16]indicating that small airway disease is reflected in the dimensions of the larger proximal airways. Also, airway dimensions as assessed by CT were shown to correlate with crucial lung function parameters like FEV1 in several reports [17,18]. Importantly, Nakano et al have demonstrated that CT image analysis for both airway dimensions and for lung density can be used for the description of COPD phenotypes [17].

This approach of CT phenotyping is currently being used in several large studies in order to discover markers associated with the emphysema and airway disease phenotypes [19,20] . Genetic markers expressed by the crucial lung cells, i.e. the alveolar macrophages and bronchial epithelial cells, are, however, not being investigated in the above studies. The EvA study uses the CT approach for definition of the patient population and it performs bronchoscopy in order to recover these cells by lavage and bronchial brush, respectively. Genes expressed in these tissues will then be tested for association with the E and A phenotypes of COPD in a quest for new targets for diagnosis and therapy.

Aims

The aim of the EvA project is to identify novel markers for COPD and its main phenotypes, i.e. emphysema and airway disease, by studying gene expression in bronchial and alveolar samples from patient lungs compared to matched controls.

Consortium structure

The EvA study is an European Union funded project (FP7, # 200506) which is bringing together ten clinical centers in five European countries, four diagnostic centers including one small to medium size enterprise (SME) and the European Respiratory Society (ERS). The EvA-Coordinator is LZH at the Helmholtz-Zentrum Muenchen and Asklepios Hospital in Muenchen Gauting. The project is managed by a steering committee and the annual meeting of the entire consortium.

Overall design

Recruitment of cases and controls to the study is done over a three year period (2/2009 to 3/2012) and the study aims for bronchoscopy in 300 controls and CT and bronchoscopy in 500 cases. Participants are invited to visit the clinical centers on three occasions ideally within a period of four to six weeks. The following investigations are done: visit one: detailed history, clinical examination, 6-min walking distance test, bioimpedance, ECG, spirometry before and after bronchodilation and clinical laboratory; visit two: CT scans and visit three: bronchoscopy with brushing of the airways and bronchoalveolar lavage of the alveoli.
Gene expression in the two types of lung tissue, i.e. bronchial epithelial cells and alveolar macrophages, is then compared to CT-defined emphysema and airway disease phenotypes.

The details of this study are given below.

Recruitment

Recruitment of cases and controls is initiated by using campaigns of small newspaper adverts and candidates are preselected by telephone interview and detailed information on study protocol and consent forms are sent by mail. Also, a multi-language webpage (www.eva-copd.eu/eva/english/) informs candidates about the details of the study. When invited to the study center participants are reminded not to take their respiratory medication on the day. Recruitment to this type of study requires quite some effort since volunteers will be subjected to extensive investigations including radiation exposure but most importantly they need to agree to undergo the invasive procedure of the bronchoscopy. Also, we have to take into account that there will be a fraction of participants, who decide late in the study not to go forward to bronchoscopy.

Initial examination at visit one

During visit one candidates willing to participate have the opportunity to discuss all aspects and informed consent is obtained in line with the Declaration of Helsinki and based on approval by the local Ethics Committees.

A clinical history including smoking pack years is taken and Medical Research Council (MRC) dyspnea score is determined. Lung function is done using body plethysmography before and after maximal bronchodilation using 4 inhalations of 100 µg of salbutamol each. Lung function parameters assessed include pO_2, pCO_2, TLCO/Va , TLC, IC, RV, FEV_1, FVC before bronchodilation and FEV1, FVC and resistance after bronchodilation. Lung function analysis will exclude many patients because of a strong asthmatic component (delta FEV1 before and after bronchodilation > 400 ml) or because their post-bronchodilator FEV1/FVC ratio is 0.7 or above such that they do not qualify as COPD. Also, stage IV cases and those with a FEV1 lower than 1 L are excluded.

The history covers general medical aspects and details of smoking habits (cigarettes only, cigar and pipe smoking is ignored) and of respiratory disease including Medical Research Council (MRC) dyspnea score. Bioimpedance measurement is taken for body composition, 6MWD test is done as part of the BODE score and after inhalation of isotonic saline solution induced sputum is recovered.

A detailed clinical examination is done and blood is taken for extensive clinical chemistry in order to document comorbidities and to identify diseases that might

affect fitness to participate. ECG is done to exclude any cardiac disease that might preclude bronchoscopy.

Exclusion criteria

While many other studies into COPD can include any stage of the disease the EvA group is different because of the invasive bronchoscopy procedure such that most severe forms of COPD with very low FEV1 and oxygen levels and patients at older age are excluded from participation (Table 1). Also, since in EvA the inflammatory process in the lung is studied we exclude current smokers in order to avoid the smoke induced inflammation. Patients with frequent exacerbations leading to hospitalization are excluded as well since the sequelae of such acute events may still impact on the inflammatory response at the time of bronchoscopy. With this approach we want to ensure that the markers, which we will identify in the COPD lung, are due to COPD and not to any other inflammatory process. We also exclude patients on oral glucocorticoids since these drugs may substantially reduce inflammation in the lung.

In order to focus on prototypic smoke induced COPD we exclude patients with low exposure (< 5 PY), excessive exposure (> 80 PY) and young age. Also, other diseases that lead to lung destruction like alpha-1-anti-trypsin deficiency and IgG deficiency are excluded. Patients with a pronounced asthmatic component (> 400 ml improvement of FEV1 post bronchodilation) are excluded since there is the possibility that the fixed obstruction in an ex-smoker is not due to COPD but is the result of chronic asthma. For control donors the same exclusion criteria apply, but asthma is allowed for.

Table 1 Exclusion criteria for EvA study

COPD Stage IV, FEV1 < 1.0 L, LTOT*, age > 75	increased risk for bronchoscopy AEs
oral glucocorticoids	may attenuate inflammation
active smokers	induces acute inflammation
frequent severe exacerbations	may interfere with CT, bronchoscopy
age < 45, < 5 PY	other causes likely
> 80 PY	low susceptibility to COPD
AAT, IgG deficiency	will mimic COPD phenotype
post-bronchodilator FEV1 increase > 400 ml	asthma as cause of fixed obstruction
serious co-morbidities, anti-coagulation	precludes bronchoscopy
cardiac pacemaker implants	generates CT artefacts
major lung surgery	lung function test, CT is distorted

* longterm oxygen therapy

CT at visit two

CT scans on COPD patients and a limited number of healthy controls (n= 30) will be done in 10 clinical centers using either General Electric or Siemens scanners. The settings and the recons are predefined such that there is minimal deviation among the different scanners.

The whole lung EvA-E scans cover the entire lung from the diaphragm recesses to the apex. This scan is used for assessment of lung density. The limited EvA-A scan includes the region from 2 cm below the carina to the top of the aorta. The limited scan will be used to determine dimensions of third generation airways like S1. All scans are done at maximum inspiration with breath holding. The recording is done from base to apex, which is important for the whole lung scan in order to avoid motion artefacts at the diaphragm at the end of the 20 sec breath-holding. Before the procedure the patients inhale 4 x 100 µg salbutamol using a spacer and after 15 minutes they are positioned in the scanner. A phantom containing three rods of different density is mounted to the chest in the sternum area.

The scans are recorded with a rotation time of 0.5 sec, a tube voltage of 120 kV, a tube current of 40 (EvA-E) or 50 mAs (EvA-A) and this dose was kept fixed for all patients [21]. The EvA-E protocol is optimal for density resolution at the expense of spatial resolution. Conversely for the EvA-A protocol pitch and speed are lower, which allows for a better spatial resolution for the airway analysis. This, however,

goes along with a higher radiation dose. In order to keep the radiation exposure low the EvA-A scan covers a limited part of the lung, (including the right S1 segmental bronchus) such that this protocol comes at a dose of 0.42 mSv only. The total radiation exposure for both scans is 2.1 mSv. Approval is obtained from the relevant radiation protection authorities.

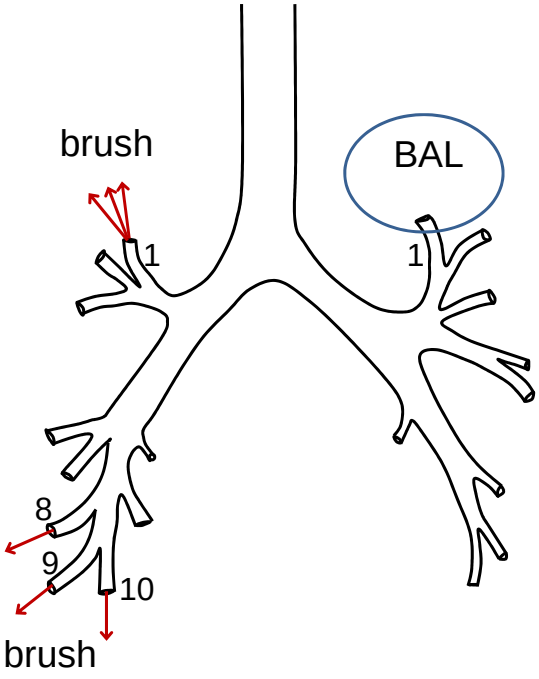
CTs are inspected for clinical reporting by the clinical radiologist on duty. Patients with nodules > 10 mm will not go forward for EvA bronchoscopy but they will be referred for further diagnostics and therapy for potential malignancy. When these findings have been taken care of then the patients can be re-entered into the EvA study. Cases with nodules < 10 mm can proceed to EvA bronchoscopy and they will be referred for further management thereafter.

The whole lung EvA- E-scans are analysed for lung density using the PULMO CMS software (Medis specials, Leiden, The Netherlands) and this is expressed as Perc15 (HU), which is the density in Hounsfield units of the lower 15th percentile of the lung. Additional read outs will be the percentage of lung tissue with different lung density cut-offs (e.g. -950HU) and the different regions of the lung will be analyzed. Correction of lung density for inspiratory level will be done using TLC derived from body plethysmography based on the “sponge model” [22].
In the limited EvA-A-scans the S1 bronchus is studied for airway wall area using Emphylx-J (University of British Columbia, Vancouver, BC, Canada). The right S1 runs almost perpendicular to the CT slices and therefore its inner and outer diameter can be assessed directly without adjustment for oblique angles. The area of the airway wall is then expressed as percentage of the entire airway area (WA (%)). In order to assess airways of any orientation 3D software packages will be used to study volumetric airway wall dimensions for a series of airways down to the 6th generation.
For normalization a set of phantoms, which provide different densities and different airway wall areas is used. The Warwick phantom was put together at the University of Coventry and Warwickshire and consists of 9 cores of polyurethane foam with different degrees of expansion held in a solid water frame. The airway phantom was generated at the University of Leicester and contains 9 teflon tubings of different sizes in a polystyrene block. A commercial phantom (CTP674, Catphan®, The

Phantom Laboratory, Salem, NF, USA) also contains tubings of different dimensions. These three phantoms plus the densitiy rods mentioned above are scanned with the EvA-E and EvA-A protocols on all 10 scanners and the readings are used to determine correction factors for both Perc15 (HU) and WA (%).
The values Perc15 (HU) and WA (%) will be used to define patients with pronounced emphysema and little or no airway wall thickening (emphysema-dominant) and patients with little or no emphysema and pronounced airway wall thickening (airway disease dominant) along the lines of the approach taken by Nakano et al [17] with the cut-off values to be defined based on the distribution of the values. Definition of the phenotypes will be assisted by testing additional relevant parameters like TLCO/Va. The phenotypes derived from the CT scan analysis will be analyzed for associations with the DNA, RNA and protein markers generated within the EvA study.

Bronchoscopy at visit three

For bronchoscopy patients are asked not to use inhaled drugs on the day. They are placed in a semi-recumbent position and receive a mild sedation via a venous access. The flexible endoscope is inserted via a nostril and the airways are inspected. Brush and lavage samples are taken from defined areas of the lung as shown in Figure 1. In order to ensure a uniform approach by the bronchoscopists a video of the procedure is produced and can be viewed by all partners on the EvA data center platform.



Legend to Figure 1: Schematic representation of the airways with the relevant segmental bronchi numbered. For the right lung the red arrows show the sites for brushing. For the left lung the blue ring indicates the preferred segment for lavage.

Brushing is done in the right lung with sheathed brushes with a diameter of 5 mm at bristle level (#BC-202D 5010, Olympus, Hamburg, Germany). After three strokes the brush is rotated in the airway 5 times and then the brush is resheathed and recovered. Three brushings are taken from the subsegments of S1 in the right upper lobe and are pooled as one sample and three brushings from the right lower lobe from subsegments of S8, S9 and/or S10 are pooled as one sample. The brushes are transferred into RNA protect (# 76526 Qiagen, Hilden, Germany) immediately. The lavage is done in the left upper lobe or lingula with a total volume of 150 ml of sterile, pyrogen-free, 37°C saline, which is instilled in 20ml fractions typically into the left S1 segment with aspiration of the volume with 20ml syringes. The lavage fluid is spun immediately and the cells are counted and transferred into RNA protect. Patients are monitored until completely recovered and are discharged after medical examination. The approach taken allows for an out-patient bronchoscopy since there is no general anesthesia and the volume for lavage is very low thereby reducing any impairment of gas exchange. At the same time it will provide two types of lung samples, i.e. bronchial epithelial cells and broncho-alveolar lavage that is composed predominantly of alveolar macrophages.

Data Storage and Quality Control

Data are all entered in a web-based central storage, based on a Cold Fusion/ Java Interface System that is linked to a H2 relational database and all entries are logged in separate files. Data management includes plausibility and range checks and protocol violations are flagged. Time-stamped data backup as well as a mirror copy of the whole system is run every night onto a second server. Data access is restricted to users identified by password while the database holds only a unique ID number for the study probands.

Marker analysis

The coded cell samples from blood and from the lung are stored at -20°C and batches are processed for DNA and RNA isolation. Quality will be assessed based on amount and on integrity of RNA of the samples. Using state of the art technology at the time the transcriptome will be determined with the aim to identify transcripts that show a differential expression between the COPD phenotypes and DNA is used for SNP analysis and for whole genome sequencing in order to assess the number and nature of acquired mutations. Also, SNPs will be tested for association with gene expression data. Fluids are analysed for proteins encoded by genes that show differential expression between COPD and controls and between the phenotypes of COPD. Also candidate proteins Eosinophil Cationic Protein (ECP), Eosinophil Protein X (EPX) and Myeloperoxidase (MPO), human neutrophil lipocalin (HNL), Lysozyme, Human Phospholipase B-II and sCEACAM8 as well as s well as neutrophil α-defensins and the cathelicidin hCAP18/ LL-37 are determined in the fluids from blood and the lung.

Markers determined at the DNA, mRNA and protein level are then tested for association with COPD and with the COPD phenotypes E and A. For the final data analysis a SQL export is being prepared and imported into R statistical analysis system where further datasets from DNA and RNA sequencing are being merged. R 2.12 is run under R Studio 09.4 including numerous libraries including R Bioconductor. For the main analysis we will do a final classification of COPD cases and controls while for the main COPD subtype analysis emphysema cases will be tested against airway disease cases. Following a baseline characteristics of cases and controls we will compute crude odds ratios for different categories of risk factors from these two 2x2 tables while for an advanced analysis of indicators of previous disease we will use multivariate linear and logistic regression models taking into

account also confounders like current treatment. More explorative analysis of bronchial and alveolar gene expression will use principal component analysis and other clustering methods to define unique profiles and biological pathways that are relevant to COPD subtypes.

Discussion

Many studies into genetics of COPD have in the past looked at the disease as one entity such that genes linked to phenotypes have evaded detection. In the EvA study we therefore define the COPD phenotypes emphysema and airway disease using CT image analysis and we search for markers associated with these as is being done in other ongoing studies into COPD [18, 19]. In addition to looking at associations with DNA markers we take samples from alveoli and airways in order to study the transcriptome in alveolar macrophages and bronchial epithelial cells, respectively. Recruitment to this type of study obviously is a challenge because of the invasive procedure, such that patients and controls, who are willing to participate in a COPD study, are reluctant to be included when it comes to bronchoscopy. Therefore for an invasive diagnostic study like EvA it is difficult to predict, whether a target for numbers of bronchoscopy can be really achieved.

The population of the EvA study is unique since there are many exclusion criteria, which are meant to a) focus on bona fide COPD to, b) avoid effects of current smoking and immunosuppression on gene expression and to c) prevent any harm to participants during bronchoscopy. The latter involves excluding patients with stage IV, FEV1 < 1L and LTOT since very low FEV1 and respiratory failure carry a higher risk for a decrease in blood oxygen levels during bronchoscopy and inclusion of such patients cannot be ethically justified when there is no medical indication for the procedure. Also comorbidities like symptomatic coronary artery disease, arrhythmias (e.g. atrial fibrillation), uncontrolled hypertension and severe liver and kidney diseases lead to exclusion.

Current smoking would introduce many artefacts including inflammation in the airways. Also, active smokers have increased numbers of alveolar macrophages, which are loaded with tar and have impaired function [23]. When we want to study genes which are regulated by the emphysema or airway disease processes in COPD, then the interference by current cigarette smoking will very likely blur the pattern. Therefore all study participants have to be ex-smokers for more than a year. It may be argued that exclusion of late stage patients with severe disease, i.e. low FEV1 and requirement of oxygen therapy, does not give the full account of the COPD

spectrum. While patients with a rapid progression of disease have been discussed to be a separate phenotype, many stage IV patients have started with mild disease and have gradually progressed. Hence they are COPD patients that are studied at a later point in time. Analysis of the transcriptome in stage IV patients may even be misleading in that hypoxia, the restricted mobility and the use oxygen therapy may impact on gene expression and mimic a unique phenotype.
Anyway, the unique composition of the patient population will always have to be taken into account, when data obtained under EvA will be compared to other studies, which allow for factors that modulate inflammation and which use a non-invasive approach. Therefore, findings of the EvA study will have to be confirmed for the entire spectrum of COPD including stage IV patients, current smokers and those older than 75.

A problem with the selection of appropriate patients is the differentiation of COPD from asthma. Fabbri et al [24] observed that the fixed airway obstruction in adults with a history of asthma can be very similar to that seen in patients with a history of COPD. When being confronted with a population of ex-smokers with a fixed airway obstruction, this population may contain cases, which always have been told to have COPD because they were smokers. Such cases may be identifiable based on high eosinophil count in sputum [22] and this parameter will be used for post-hoc exclusion in the EvA study.
One challenge of the project is that scans obtained with different scanners (General Electric and Siemens) and different makes are used by the EvA partners and it is important to standardize with the use of phantoms. For this, standardization against air, standard density rods and a series of newly generated phantoms are be used. We expect this strategy to substantially improve data interpretation in this multicenter study. For determination of the degree of emphysema and of airway wall thickening the study protocol calls for two different dedicated CT scans. While the scan for airway wall area provides a higher resolution, it is also possible to determine dimensions of multiple airways using the lower resolution scans of the whole lung, i.e. this scan might be used for assessment of both emphysema and airway disease.

The bronchoscopy will be done with a flexible instrument and with mild sedation such that participants need not stay in hospital overnight. This is a safe routine procedure,

which, however, carries as any invasive procedure the risk of infection and bleeding plus as a rare event a pneumothorax. Therefore not only ethics approval but also insurance cover is required for the study. The brushing to obtain airway epithelial cells will target the right lung and here specifically the S1 bronchus with its subsegments (see figure 1). This type of sample is expected to be highly informative since it is taken from the very site, for which we determine the airway wall thickness in CT image analysis. It will be exciting to correlate the thickness of the airways in this area with the gene expression by the epithelial cells obtained from S1.

The EvA study is designed such that samples from the lung are collected over a three year period of time. Only when the collection is complete then the molecular analysis will be done in large batches. The specific platform and the depth of analysis will be decided at the time based on the technology available.

The EvA study is taking approaches that are distinct to other ongoing studies. ECLIPSE is a longitudinal study in a group of over 2000 patients that aims to identify markers and parameters that correlate with clinically relevant COPD subtypes including CT phenotypes and predict disease progression [19] Biomarkers are assessed in blood, urine, sputum and exhaled breath. In comparison, the EvA study with 500 patients is cross-sectional and in addition to sampling of blood and sputum a bronchoscopy is performed and airway brush samples and bronchoalveolar lavage samples are recovered. Therefore, the EvA study is closer to main organ affected by COPD and it aims at identifying markers expressed in the lung tissue at the DNA, RNA and protein level.

COPDGene is a genome-wide association study aiming for 10 000 patients [20]. Similar to EvA it will determine the degree of emphysema and airway wall thickening using CT scans. DNA extracted from blood is then used to run SNP arrays and associations are determined with parameters of emphysema and airway wall thickening taken from the CT scan analysis. By contrast in the EvA study associations with SNPs is only a secondary aim and will be restricted to confirmation of known associations. COPDGene is restricted to analysis of blood while EvA focusses on gene expression in the lung. The number of patients enrolled into EvA is low compared to the above studies. This may be counterbalanced by the stringent

exclusion criteria (see Table 1), which result in a more clearly defined patient population with for instance little contamination by asthma cases. In addition power calculations have shown that the study is sufficiently powered to detect a 20% difference between E and A (2-tailed at 5% alpha error is 94.6%).

Taken together EvA is a Europe-wide case control study into COPD, which aims at defining COPD phenotypes by CT image analysis and which puts an emphasis on the analysis of gene expression in tissue samples taken from the lung. We expect this approach to provide exciting new data on novel markers that can be used for diagnosis of and as target for therapy of COPD phenotypes.

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